

Microbial biotechnology

Rapid Advances in an area of massive impact

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Abstract

For thousands of years, microorganisms have been used to supply products such as bread, beer and wine. A second phase of traditional microbial biotechnology began during World War I and resulted in the development of the acetone-butanol and glycerol fermentations, followed by processes yielding, for example, citric acid, vitamins and antibiotics. In the early 1970s, traditional industrial microbiology was merged with molecular biology to yield more than 40 biopharmaceutical products, such as erythropoietin, human growth hormone and interferons. Today, microbiology is a major participant in global industry, especially in the pharmaceutical, food and chemical industries.

Key words: Primary metabolites, Secondary metabolites, Recombinant DNA technology.

Introduction

Microorganisms are important for many reasons, particularly because they produce things that are of value to us (Demain, 1990). These can be very large materials (e.g. proteins, nucleic acids, carbohydrate polymers, even cells) or smaller molecules and are usually divided into metabolites that are essential for vegetative growth (primary) and those that are nonessential (secondary). Microbial technology has made significant advances in recent years with an overwhelming impact on the society. The developments are very fast and new dimensions are being added every day. Microbial technology explores and exploits the microbial wealth for human requirement like production of microbial metabolites and products such as enzymes, organic acids, antibiotics, drugs and pharmaceuticals, in processes like recombinant protein expression, fermentation and downstream processing and in bioremediation, bioleaching, soil and waste management etc.

On the other hand, there is a great deal of microbe-based biotechnological

development and micro-organisms have become indispensable tools in molecular biology, genetic engineering and DNA technology research. Many of these advances are revolutionising medicine and paradoxically several pathogenic microbes. The twenty-first century belongs to the gene era. The illuminating ideas of genetic fundamentals are making modern biology the fastest growing and most exciting area of science and technology. The isolation of genes and their sequencing are now a routine phenomenon. The isolated genes can be multiplied and mutated at will. The altered gene can be introduced into host cells to investigate its effect on phenotype or to assign specific function. It is also now possible to synthesize genes based on their known nucleotide sequences. The landmark break has been the determination of the human genome sequence, which has given impetus to the disciplines of genomics, proteomics, pharmacogenomics, structural biology and bioinformatics. Of the vast range of exciting research areas, priorities for concerted attention would naturally be those with practicable objectives either realizable immediately or in the near future which could

contribute to the economic growth of the country and to the protection and improvement of human health. From this point of view, some of the promising areas of research and development are outlined below

Primary metabolites

Primary metabolites are the small molecules of living cells; they are intermediates or end products of the pathways of intermediary metabolism, building blocks for essential macromolecules, or are converted into Coenzymes. Primary metabolites used in the food and feed industries include: alcohols (ethanol), amino acids (monosodium glutamate, lysine, threonine, phenylalanine, tryptophan), flavor nucleotides (5-guanylic acid, 5-inosinic acid), organic acids (acetic, propionic, succinic, fumaric, lactic), polyols (glycerol, mannitol, erythritol, xylitol), polysaccharides (xanthan, gellan), sugars (fructose, ribose, sorbose) and vitamins [riboflavin (B_2), cyanocobalamin (B_{12}), biotin (Bu Lock *et al.*, 1965).

Secondary metabolites

The best-known groups of the secondary metabolites are the antibiotics (Strohl, 1997). Their targets include DNA replication (actinomycin, bleomycin and griseofulvin), transcription (rifamycin), translation by 70-S ribosomes (chloramphenicol, tetracycline, lincomycin, erythromycin and streptomycin), transcription by 80-S ribosomes (cyclohexamide), transcription by 70- and 80-S ribosomes (puromycin and fusidic acid), cell wall synthesis (cycloserine, bacitracin, penicillin, cephalosporin and vancomycin) and cell membranes (surfactants including: polymyxin and amphotericin; channel-forming ionophores, such as linear

gramicidin; and mobile carrier ionophores, such as monensin). There are other secondary metabolites (SM) with an enormous range of other biological applications mainly in the field of pharmaceuticals and cosmetics, food, agriculture and farming (Demain, 1990). These include compounds with anti-inflammatory, hypotensive, antitumour, anticholesterolemic actions and also insecticides, plant growth regulators and environmental friendly herbicides and pesticides.

Since 1940, there has been a virtual explosion of new and potent antibiotic molecules that have been of great use in medicine, agriculture and basic research. In 1996, the antibiotic market was composed of 160 antibiotics and amounted to a world market value of ~US\$23 billion. The search for new antibiotics continues, in order to: combat evolving pathogens, naturally resistant bacteria and fungi, and previously susceptible microbes that have developed resistance; improve pharmacological properties; combat tumors, viruses and parasites; and discover safer, more potent and broader spectrum compounds. In the search for new antibiotics, many of the new products are made chemically by modification of natural antibiotics via semisynthesis. Antibiotics are used not only for chemotherapy in human and veterinary medicine, but also for growth promotion in farm animals and for the protection of plants (Campbell, 1989).

Alcohol

Ethyl alcohol is a primary metabolite produced by fermentation of sugar, or a polysaccharide that can be depolymerized to a fermentable sugar. *Saccharomyces cerevisiae* is used for the fermentation of hexoses, whereas *Kluyveromyces fragilis* or *Candida* species can be used if lactose or a pentose, respectively, is the substrate. Under optimum conditions, approximately 1012% ethanol by volume are obtained within five days. Such a high concentration slows down growth and the fermentation ceases produce alcohol concentrations of 20% by volume, but these concentrations are attained only after months or years of fermentation.

At present, all beverage alcohol is made by fermentation. Industrial ethanol is mainly manufactured by fermentation, but some is produced from ethylene by the petrochemical industry. Bacteria such as clostridia and *Zymomonas* are being re-examined for ethanol production after years of neglect. *Clostridium thermocellum*, an anaerobic thermophile, can convert waste cellulose (i.e. biomass) and crystalline cellulose directly to ethanol.

Other clostridia produce acetate, lactate, acetone and butanol, and will be used to produce these chemicals when the gobbie petroleum supplies begin to become depleted. Fuel ethanol produced from biomass would provide relief from air pollution caused by the use of Gasoline and would not contribute to the greenhouse effect. *E. coli* has been converted into an excellent ethanol producer (43% yield, v/v) by recombinant DNA techniques.

Recombinant DNA technology

Recombinant DNA technology is beginning to have a major impact on amino acid production such as Threonine, Isoleucine, Leucine, Valine, Phenylalanine, Tryptophan, Tyrosine, Proline, Arginine, and Histidine (Jetten and Sinskey, 1995; Sahm *et al.*, 1995). The advent of recombinant DNA technology has extended the range of potential microbial fermentation products. It is possible to introduce genes from higher organism into microbial cells such that the recipient cells are capable of synthesizing foreign proteins. Example of the host for such foreign genes include *Escherichia coli* and *Saccharomyces cerevisiae* and other yeasts as well as filamentous fungi such as *Aspergillus niger* var. *Awamori*.

Products produced in such genetically manipulated organisms include interferon, insulin, human serum albumin factor VIII and factor IX epidermal growth factor, bovine somatostatin and bovine chymosin (Harris, 1990; Wiseman, 1991). Important factors in the design of these processes include the secretion of the product, minimization of the degradation of the product, and the control of

the onset of synthesis during the fermentation, as well as maximizing the expression of the foreign gene (Hockney, 1994).

A general process for the production of rDNA products is illustrated in Figure 1. The first step is isolation of the identified gene that is responsible for expression of the desired product. After isolation and characterization of the human gene, it is inserted into small circular pieces of DNA called plasmid. The recombinant plasmid is inserted into bacterial yeast or cultured animal cell. Clones of transformed host cell are isolated and those that produced the protein of interest in the desired quantities are preserved under suitable condition as a master cell bank. The cell banks are characterized and properly maintained for use in subsequent transformation procedures. The cell bank should be periodically tested for cell viability, genetic and phenotypic stability. As manufacturing needs arise, cells from working cell can be scaled up to produce the product in roller bottles and/or fermentors.

Enzyme production

The production of enzymes by fermentation was an established business before modern microbial biotechnology. However, recombinant DNA methodology was so perfectly suited to the improvement of enzyme production technology that it was almost immediately used by companies involved in manufacturing enzymes. Industrial enzymes have now reached an annual market of US\$1.6 billion. The enzyme industry is one among the major industries of the world and there exists a great market for enzymes in general. Pharmaceutical industry is being recognized as an important consumer for commercial enzymes. Enzymes are in great demand for use as therapeutic agents against many dreaded diseases. Accelerated and in depth studies to utilize the vast microbial resources-both terrestrial and marine - as sources of novel therapeutic enzymes are highly significant. Microbial enzymes offer the potential to treat many important diseases, which are resurging after acquiring resistance to antibiotics. Some of these are listed in (Table-1)

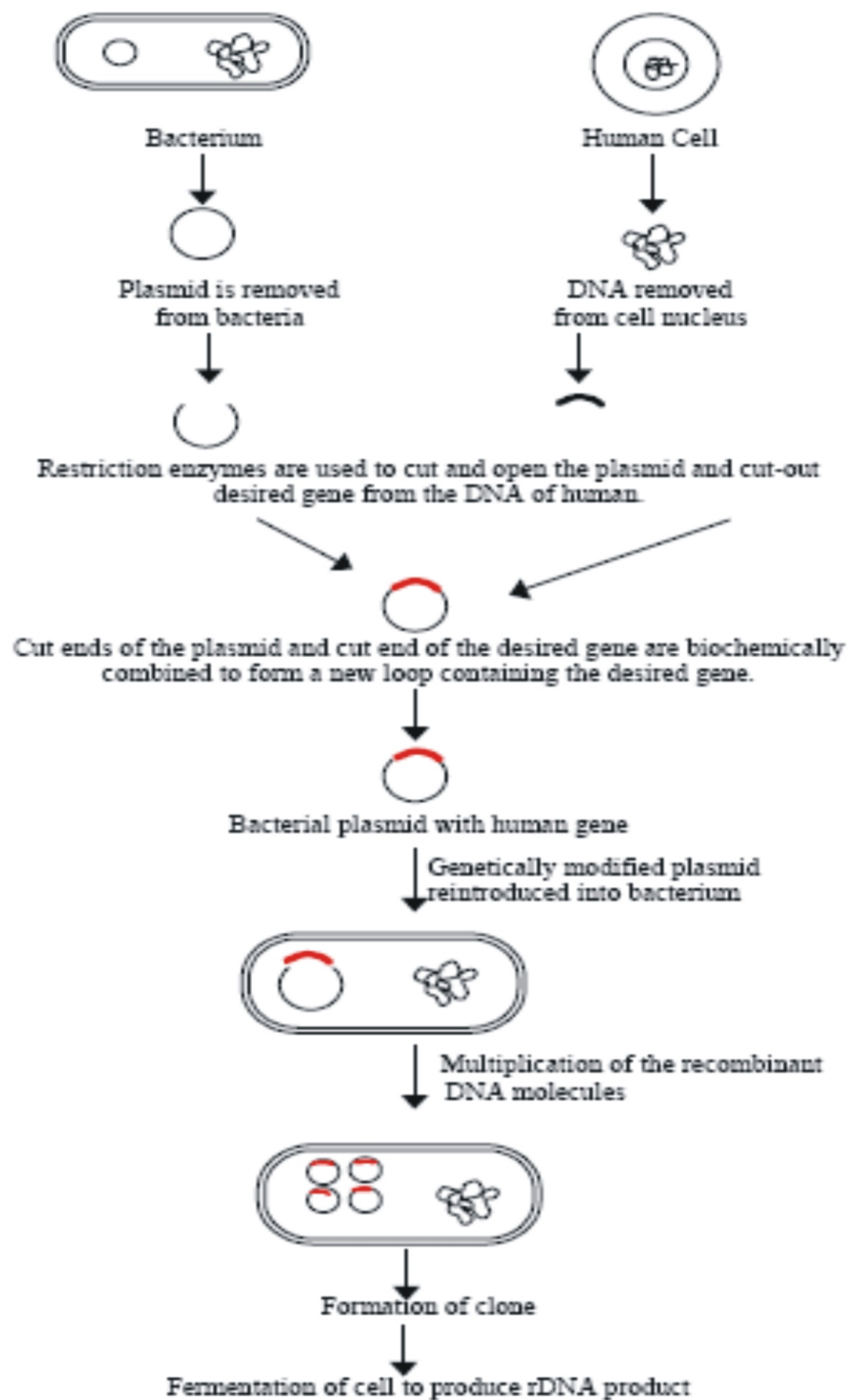


Figure 1. Recombinant DNA technology for production of human therapeutics.

Table 1 - Some important therapeutic enzymes and their applications

Streptkinase	-	Anticoagulant
Urokinase	-	Anticoagulant
L-Asperginase	-	Anti tumor
L-Glutaminase	-	Anti tumor
Superoxide dismutase	-	Anti-oxidant, anti-inflammatory
Penicillin acylase	-	Synthetic antibiotic production to treat skin ulcers
Collagenase	-	
Lipase	-	Digest lipids
Ribo nuclease	-	Antiviral

Enzymes of microbial origin are preferred to those from plants because of the economy in production and the consistency and ease of process modification and optimization. These enzymes are relatively more stable. The advent of rDNA technology allows production of large quantities of pharmaceutical proteins, which were previously difficult and costly to produce. So far four hundred human proteins have been produced by rDNA technology for therapeutic use. Important enzymes are proteases, lipases, carbohydrases, recombinant chymosin for cheese manufacture and recombinant lipase for use in detergents. Recombinant therapeutic enzymes already have a market value of over US\$2 billion, being used for thromboses, gastrointestinal and rheumatic disorders, metabolic diseases and cancer. They include tissue plasminogen activator, human DNAase and Cerozyme. Their commercial value is enormous and the present global market for therapeutic recombinant proteins is around \$200 billion. Commercially available FDA approved recombinant human proteins (Table-2)

industry etc. Strain improvement of microorganisms in food products has been difficult as isolation and mutation are time consuming and labor intensive. Food fermentation applications such as in dairy products and alcoholic beverages have also shown good possibility for using genetic modifications for improved fermentation performance and resistance to bacteriophages rather than yield improvement. Improvement in product characteristics including better nutritive quality will be the driving force of future research in food biotechnology (Hilmer Nielsen, 1980).

Bioconverting-organisms

In addition to the multireaction sequences of fermentations, microorganisms are extremely useful in carrying out biotransformation processes in which a compound is converted into a structurally related product by one or a small number of enzymes contained in the cells (Kieslich, 1997). Bioconverting-organisms are known for practically every type of chemical reaction. The reactions are stereo specific; the ultimate in specificity is

Table 2 - Commercially available FDA approved recombinant human proteins

Recombinant protein	Manufacturer	Application
DNase 1	Genentech	Cystic fibrosis
Drythro poietin	Amgen	Anemia
Growth hormones	Genentech	Growth Hormones
		Deficiency
Insulin	Eli Lilly	Diabetes
IFN-a2a	Hoffmann-1a Roche	Leukemia
Interleukin-2	Chiron	Renal carcinoma

Enzymes have been used in food processing such as leavening of bread, fermentation of fruit juices or malt, clotting of milk for cheese etc. The world market for enzymes, mostly microbial enzymes, is over 1.5 billion dollars. Newer applications for enzymes are in detergents, textiles, paper and pulp, chemical

exemplified by steroid bioconversions. Bioconversions are characterized by extremely high yields, approximately 90-100%. Other attributes include mild reaction conditions and the coupling of reactions, using a Microorganism containing several enzymes working in series.

Agricultural biotechnology and Pest control

Industrial microbiology through genetic engineering and its associated disciplines has brought about a revolution in agriculture. Two bacteria have had a major influence: *Agrobacterium tumefaciens*, a bacterium that normally produces crown gall tumors on dicotyledonous plants; and *Bacillus thuringiensis*, an insecticidal bacterium. The tumor-forming genes of *A. tumefaciens* are present on its tumor-inducing (Ti) plasmid, along with genes directing the plant to form opines (nutritional factors required by the bacterium that it cannot produce by itself). The Ti vector has been exceedingly valuable for introducing foreign genes into dicotyledonous plants for production of transgenic plants. However, the Ti plasmid is not very successful for transferring genes into monocotyledonous plants, a problem bypassed by, for example, and the development of a particle acceleration gun, which shoots DNA-coated metal particles into plant cells.

The activity of the insecticidal bacterium, *B. thuringiensis*, is caused by its crystal protein produced during sporulation. Crystals and spores have been applied to plants for many years to protect them against lepidopteran insects. Characterizing *Bacillus thuringiensis* (Bt) strains based on cry gene contexts provides better focus on its application potential. In the recent past, organizing the cry gene nomenclature based on protein/nucleotide sequence has overcome many ambiguities that persisted for long. *B. thuringiensis* preparations are highly potent, approximately 300 times more active on a molar basis than synthetic pyrethroids and 80,000 times more active than organophosphate insecticides. After weighing the advantages and disadvantages of application of Bt in the environment, it could be concluded that Bt definitely is vastly superior to chemical insecticides, being biodegradable, nontoxic, target specific and, most importantly, renewable (Schuler, 1998).

In the modern biotechnology era, plants resistant to insects have been produced by expressing forms of the *B. thuringiensis* toxin gene in the plant. Recently developed

bioinsecticides include insect viruses, such as baculoviruses, that are engineered to produce arthropod toxins. Transgenic plants, resistant to herbicides, are also available, as are virus-resistant plants produced by expressing viral-coat-protein genes in plants. Interestingly, chemical pesticides against plant viruses were never available. Also in commercial or near-commercial use are biopesticides, including biofungicides (e.g. kasugamycin, polyoxins), bioinsecticides (nikkomycin, spinosyns), bioherbicides (bialaphos), antihelminthics (ivermectin), coccidiostats, ruminant-growth promoters (monensin, lasalocid, salinomycin), plant-growth regulators (gibberellins), immunosuppressants for organ transplants (cyclosporin A, FK-506, rapamycin), anabolic agents in farm animals (zearelanone), uterocontractants (ergot alkaloids) and antitumor agents (doxorubicin, daunorubicin, mitomycin, bleomycin).

Microbial pigments

Use of microbial pigments in processed food is an area of promise with large economic potential. However, microbial pigments offer challenges due to high cost, lower stability and variation in shades due to changes in pH. At present, none of the microbial pigments can replace synthetic pigment. The microorganisms such as *Monascus*, *Rhodotorula*, *Bacillus*, *Achromobacter*, *Yarrowia* and *Phaffia* produce a large number of pigments. An ideal pigment-producing microorganism should be capable of using a wide range of C and N sources, have tolerance to pH, temperature and minerals, and give reasonable colour yield. Non-toxic and non-pathogenic nature of pigment-producing microorganisms coupled with easy separation from the cell biomass is stressed. The various advantages of producing pigments from microorganisms include independence from weather conditions, easy and fast growth, colours of different shades and growth on cheap substances. Studies revealed unstable, largely degradable and sensitive to heat, light, acidity and water activity as characteristics of microbial colour. Improvement in stability, safety and solubility can certainly make widespread use of microbial pigments in the food industry (Joshi *et al.*, 2003)

Food Biotechnology

Microorganisms have been used for preparing food products like bread, yoghurt or curd, alcoholic beverages, cheese etc., for a long time without people knowing their involvement in fermentation. Louis Pasteur showed the role of microorganism in spoilage and subsequent elucidation and that fermentation also involves microorganisms. Some species are useful for development of flavor unique to certain wines. Citric acid is the most important organic acid produced by fermentation with an estimated annual production of about half a million tones with a value of more than half a billion dollars. It is primarily used in foods. Citric acid has been prepared from citrus fruits like Lemon but now it is mostly produced by fermentation using *Aspergillus Niger* in large corrosion resistant fermentors having stirrers. Lactic acid is another important acid produced by fermentation and mostly used for the manufacture of emulsifiers and additives in food industry. Strain improvement of microorganisms in food products has been slow as isolation and mutation are time-consuming and labour-intensive. Hybridization also is slow as unwanted traits have to be bred out. Applications with food related enzymes were the first products of modern biotechnology, followed by organic acids and amino acid production by microorganisms. Food fermentation applications such as fermented dairy products and alcoholic beverages have also shown good possibility for using GMOs for improved fermentation performance and resistance to bacteriophages rather than yield improvement. Improvement in product characteristics including better nutritive quality will be the driving force of future research in food biotechnology (Pai, 2003).

Vaccines

The approach to vaccine development has undergone remarkable progress since 18th and 19th centuries, when Edward Jenner and Louis Pasteur pioneered the use of attenuated and inactivated vaccines. The conventional approach to vaccine development requires cultivation of the pathogen and its dissection using biochemical, immunological and

microbiological methods. Although, successful in several cases the method is time consuming and has failed to provide a solution for many human pathogens. The first subunit vaccine on the market was that of hepatitis B virus surface antigen produced in yeast. The great contribution made by recombinant vaccines is the elimination of the tragic problem associated with conventional vaccines. Now genomic approaches allow for the design of vaccines starting from the prediction of all antigens *In silico*, (that is, in the computer using bioinformatics methods) independently of their abundance and without the need to grow the microorganisms *in vitro*. A new strategy, termed 'Reverse Vaccinology', which has been successfully applied in the last few years, has revolutionized the approach to vaccine research.

The possibility of determining the complete genome sequence of a bacterium in a few months at low cost enabled the sequencing of the genomes of most bacterial pathogens in a short period of time. Today, databases contain the complete genomic sequences of more than 80 bacteria, including most bacterial pathogens. More than a hundred additional bacterial genomes are in the process of being sequenced. Large genomes of parasites such as malaria have been sequenced. Powerful technologies such as analysis, Proteomics (two-dimensional gel electrophoresis and mass spectrometry), DNA micro arrays, *In vivo* expression technology (IVET) and Signature tagged mutagenesis (STM) have revolutionized the way of study of bacterial pathogenesis and vaccine design.

This is just as well, because in the context of frequent rise of new disease in epidemic form, (SARS, HVC, HIV, Borel Fly etc.), dramatic surge in pandemics, and mutation and development of drug resistance in pathogens, it is vital for mankind to have ready response systems, to identify the sequence and develop antibodies and drugs. The emergence of SARS on the global health stage early in 2003 was in some ways perhaps the most dramatic of all, sudden epidemic outbursts. Its rapid containment is one of the biggest success stories in public health in recent years.

Antiviral drugs

The antiviral era is upon us, with an array of virus fighting drugs on the market or under development. Genomics has been a springboard for the development of whole new classes of antiviral drugs. The majority of antiviral drugs on sale these days take aim at HIV, herpes viruses and hepatitis B and C viruses. It may be some time before virtually all the viruses become either preventable by vaccines or treatable by some effective drug therapy.

The biodrug concept involves the use of orally administered recombinant microorganisms as a new drug delivery route to prevent or treat disease. The tools used for genetic engineering that have been developed to date have led to the emergence of novel application using genetically modified organisms to produce drugs in large scale bioprocesses. An innovative extension of these approaches is drug production directly in the digestive environment by ingested living recombinant microorganisms. For this purpose, the use of recombinant lactic acid bacteria, has been studied, Yeast is also a convenient host and a good alternative for the production of bio drug.

Fungi

Filamentous fungi are widely used for the commercial production of organic acids, for example, 1 billion pounds of citric acid are produced per year with a market value of US\$1.4 billion. Citric acid is produced via the Embden-Meyerhof pathway and the first step of the tricarboxylic acid cycle; the control of the process involves the inhibition of phosphofructokinase by citric acid. The commercial process uses *Aspergillus niger* in media deficient in iron and manganese. A high level of citric acid production is also associated with a high intracellular concentration of fructose 2,6-biphosphate, an activator of glycolysis.

Other factors contributing to high citric acid production are the inhibition of isocitrate dehydrogenase by citric acid and the low optimum pH (1.72.0). Higher pH values (e.g. 3.0) lead to the production of oxalic and gluconic acids, instead of citric acid. Alternative processes have been developed

for the production of citric acid by *Candida* yeasts, especially from hydrocarbons.

Modern microbial biotechnology

Modern biotechnology is now over 25 years old (Cohen, 1979). In 1972, the birth of recombinant DNA technology propelled biotechnology to new heights and led to the establishment of a new industry. In addition to recombinant DNA technology, modern microbial biotechnology encompasses fermentation, microbial physiology, high-throughput screening for novel metabolites and strain improvement, bioreactor design and downstream processing, cell immobilization (enzyme engineering), cell fusion, metabolic engineering, bioreactor design, downstream processing, *in vitro* mutagenesis (protein engineering) and directed evolution of enzymes (applied molecular evolution).

Recombinant microorganisms

The revolutionary exploitation of microbial genetic discoveries in the 1970s, 1980s and 1990s depended heavily upon the solid structure of industrial microbiology, described above. The major microbial hosts for production of recombinant proteins are *E. coli*, *B. subtilis*, *S. cerevisiae*, *Pichia pastoris*, *Hansenula polymorpha* and *Aspergillus niger*. The use of recombinant microorganisms provided the techniques and experience necessary for the successful application of higher organisms, such as mammalian and insect cell culture, and transgenic animals and plants as hosts for the production of glycosylated recombinant proteins.

Strain improvement

The science and technology of manipulating and improving microbial strains, in order to enhance their metabolic capacities in biotechnological applications, are referred to as strain improvement. Continuous improvement of production strains is vital to success in making and keeping the fermentation and other metabolite producing industries viable. Strain improvement is

usually aimed at increasing yields of the desired metabolites (Macdonald and Holt, 1976). However, other strain characteristics can also be improved such as the removal of unwanted co metabolites. Today, strain improvement can be performed by two alternative strategies:

Classical genetic methods (including genetic recombination) and Molecular genetic methods.

Classical genetic methods:

Mutation followed by random screening.

Rational selection including genetic recombination.

Molecular genetic methods

The completion of numerous genome projects has generated a great technical potential for DNA sequencing. A part of this capacity is now being directed to sequencing the genomes of model micro-organisms. The complete genomes of *E.coli*, the yeast *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, and of 50 other micro-organisms, have already been sequenced and the sequencing of the fungi *Aspergillus nidulans* and *Neurospora crassa* is in progress. After this group, the turn is for micro-organisms of industrial importance (It is visualized that in the near future, genomics will also be applied to industrial strain development. In the near future, a number of genetic and molecular genetic methods (based on genomes) will be available to improve fermentation product yields and other strain characteristics (Chang *et al*, 1982; Windon *et al*, 1980).

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